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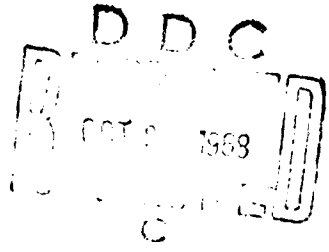

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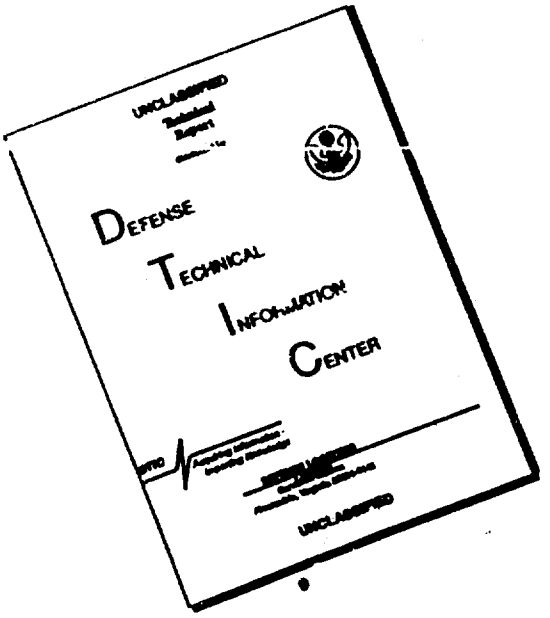

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USE OF THE H-T EXCHANGE METHOD IN STUDYING LATENT DAMAGE IN IRRADIATED TRYPSIN

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A considerable portion of the thermal effect in irradiated trypsin solutions may be ascribed to increased thermolability in the protein molecules through local modifications in structure [1]. This fact has been observed only indirectly from a modification in the kinetics of thermal denaturation. We have undertaken to observe directly the difference in structure between natural molecules of trypsin and molecules from a fraction of the ensyme with latent damage deriving from ultraviolet irradiation.

One of the most sensitive methods for detecting structural changes in protein molecules [2] is that of studying the kinetics of isotope exchange: protium - deuterium [3;4] or protium - tritium [5;6].

The first part of our experiment consisted in isolating an active trypsin fraction which had been irradiated with ultraviolet light and contained both latent damage (on the basis of kinetic data) and natural trypsin [7]. In order to study protium-deuterium exchange kinetics we had to perform lyophylic drying of the solution containing the active fraction [2]. We found that under these conditions a large part of the trypsin with latent damage was inactivated. This turned us to studying the kinetics of protium - tritium exchange by using gel filtration on sefadex G-25 [7] to separate radioactive (tritium) water from the trypsin containing tritium atoms [6]. This is a good method in that a highly accurate experiment does not require high protein concentrations. It is somewhat limited through its inability to determine the total number of exchanged hydrogen ions in the protein because of reversible exchange with the non-active water on the sefadex [6]. When experimental conditions are carefully maintained, results can be reproduced with an error factor of $\pm 4\%$. This has been verified by us in studying the kinetics of isotope exchange in trypsin and in repeating Englander's results with crystallic ribonuclease [6].

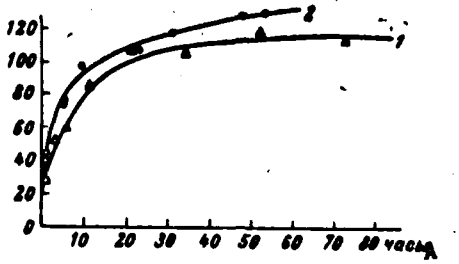

Fig. 1 Kinetics of exchange in natural trypsin (1) and an enzyme mixture containing natural trypsin and latent damage (2). Concentration - 1.38 and 1.30 mg/ml respectively, 5 M NaCl, pH 3.0
Y axis - number of tritium atoms exchanged in a single trypsin molecule
Key: A - hours.

The results of our experiment are given in Figure 1. The following procedure was used in separating irradiation-inactivated trypsin from the active fraction. A solution of the crystallic enzyme with an activity of 7000 units (VAEE) in 10^{-3}n. H_2SO_4 (C = 3.86 mg/ml) was irradiated with whole light from a PRK-2 bulb until a loss of 53% of enzymatic activity was reached. The inactivated fraction was removed by salting out in 5 M NaCl [8]. The remaining fraction, judging from kinetic data [7], must contain 25% natural and 75% latent-damage trypsin. The enzyme concentration in the solution was measured. Then a solution of natural trypsin of the same concentration was prepared. This condition must be adhered to since the nature of the kinetic line (Fig. 2, a) depended substantially on the protein concentration in radioactive water. To the solutions of natural and irradiated trypsin thus obtained we added like quantities of tritium water (in a 1:10 ratio) with an activity of approximately 20 microcurie/ml. Then these solutions were poured out in 2 series of ampoules which were kept at 3 - 4°. At certain specific time intervals samples from the ampoules (1 milliliter each) were passed through a silicon column with sefadex G-25 (3x4 cm). We found total separation of the tritium-tagged protein and the active water (Fig. 2, b). Eluent samples with the maximal protein content (Fig. 2, b) were used to measure the number of hydrogen atoms per protein molecule exchanged for tritium atoms [6]. The measurements were made with the 6012 liquid scintillation counter (IsotopeDevelopment, Ltd, England) and the SPh-4 spectrophotometer. The scintillation counter included the following components: 60 grams of sublimated naphthalene, 100 milliliters of absolute methanol, 20 milliliters of ethyleneglycol, 0,18 gram 2-alpha-naphthyl-5-phenyloxasole, and 1 liter of purified dioxane [9]. During the course of measurements 0.5 milliliter of the protein solution containing tritium was added to 5 milliliters of the scintillator solution. The number of hydrogen atoms exchanged per protein molecule was computed from a well-known formula [6]. The molecular weight of trypsin was taken as 24,000.

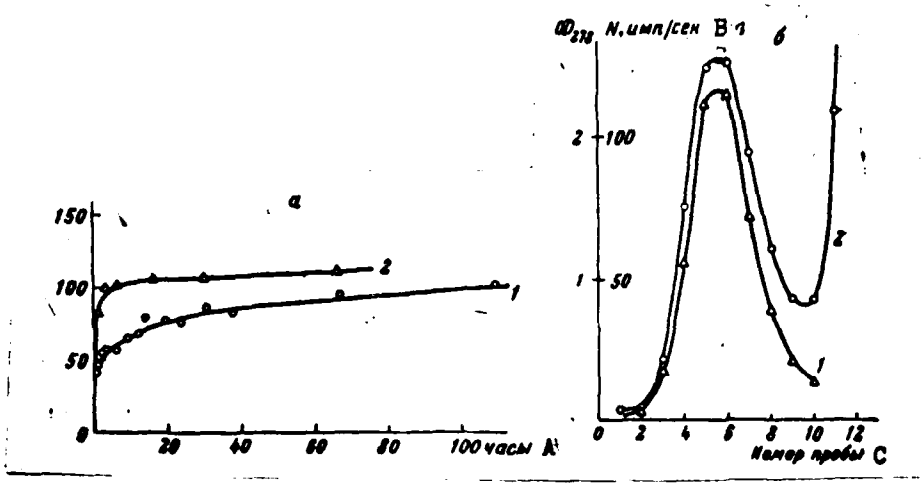

Fig. 2 a - exchange kinetics of natural trypsin as a function of concentration 10^{-3}n. H_2SO_4

1 - concentration 7.0 mg/ml; 2 - concentration 1.4 mg/ml
y axis - number of tritium atoms exchanged in one trypsin molecule.

b - separation of tritium-tagged trypsin from water containing HTO on sefadex G-25 (average, 3x4cm)
1 - optical density at 278 millimicrons
2 - intensity of tritium count

KEY: A - hours B - impulses per second C - sample number

In Fig. 1 we can see that the kinetics of isotope exchange for natural trypsin was somewhat different than that of protein with latent damage under identical conditions. The experiments described above were performed at pH 3.0 (dilute H_2SO_4). At this value the trypsin is at maximum stability. A study of hydrogen atom exchange in trypsin for tritium at various pH values showed that the number of slowly exchanged atoms (cf. above) decreases uniformly as the pH increases. Hence it follows that the maximum sensitivity of the experiment will be found at acidic readings for pH and the selected pH value is optimal.

The investigation which we made showed that the method used is the most convenient for studying structural changes in protein molecules with latent damage. Preliminary experimental data give reason for assuming that the differences observed in the kinetics of isotope exchange can be explained by certain changes in the structure of trypsin molecules with latent damage as compared with molecules of natural trypsin. However definitive conclusions can be made after the experiments have been repeated with chromatographically homogeneous protein.

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